SECURITY CLASSIF

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ON PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY UNCLASSIFIED	3 DISTRIBUTION/AVAILABILITY OF REPORT
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE UNCLASSIFIED	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION DEP APPL BIOCHEM 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Walter Reed Army Inst. of Research
6c. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100	7b. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Ft Detrick, Frederick, MD 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) US Army Medical Res & Dev Command Ft Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO

11. TITLE (Include Security Classification)
LEISHMANIA BRAZILIENSIS PANAMENSIS: INCREASED INFECTIVITY RESULTING FROM HEAT SHOCK

12. PERSONAL AUTHOR(S)
R.M. SMEJKAL, R.W. WOLFF, AND J.G. OLENICK

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Manuscript	FROM _____ TO _____		

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

DISTRIBUTION STATEMENT A
Approved for public release;
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20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21 ABSTRACT SECURITY CLASSIFICATION
22a. NAME OF RESPONSIBLE INDIVIDUAL RUTHANN M. SMEJKAL	22b TELEPHONE (Include Area Code) / 22c OFFICE SYMBOL

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

EXPERIMENTAL PARASITOLOGY 65, 1–9 (1988)

Leishmania braziliensis panamensis: Increased Infectivity Resulting from Heat Shock

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(Accepted for publication 27 April 1987)

SMEJKAL, R. M., WOLFF, R., AND OLENICK, J. G. 1988. *Leishmania braziliensis panamensis:* Increased infectivity resulting from heat shock. *Experimental Parasitology* 65, 1–9. Promastigotes of *Leishmania braziliensis panamensis* were subjected to a heat shock transformation yielding an amastigote-like stage. During the process of conversion, the heat-induced differentiating form displayed an increase in infectivity (as determined by lesion size) accompanied by a total protein composition unlike that of the promastigote and a morphology resembling that of the amastigote. These biological/functional changes may be related to an involvement of a heat shock response in the differentiation of leishmania, thus having important implications in the development of prevention and treatment stratagems. © 1988 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania braziliensis panamensis;* Protozoa, parasitic; Hemoflagellate; Heat shock; Infectivity; Heat-induced differentiating form; Developmental change; Morphology; Dulbecco's phosphate-buffered saline (PBS); Sodium dodecyl sulfate (SDS); Polyacrylamide gel electrophoresis (PAGE); Heat shock protein (Hsp).

INTRODUCTION

Leishmania spp., the causative agents of a wide spectrum of diseases, are protozoan parasites whose life cycle requires them to live and multiply under extremes of environmental conditions. The promastigote form is a slender, motile flagellate found in nature in the alimentary tract of the sandfly vector at a temperature approaching that of ambient air (Killick-Kendrick 1979; Lainson 1982). During a blood meal, promastigotes from an infected sandfly are transmitted to the warmer environment of the bloodstream of the mammalian host. Once in the host, the parasites are engulfed by macrophages, and the round, amotile intracellular amastigote form is found inside the phagolysosomes of these macrophages (Mauel 1984; Pearson *et al.* 1983; Sadick and Raff 1985).

A great deal of recent effort in leishmania research has centered on determining how and where the promastigote is transformed into the amastigote form: in the bloodstream or inside the macrophage. The amastigote is more resistant to destruction by the macrophage than is the promastigote (Mauel 1984). Therefore, determining the trigger of the promastigote to amastigote transformation is potentially of great importance in developing protective strategies.

One possible trigger is the temperature shift that the parasite undergoes when transferred from the sandfly vector to the mammalian host. In response to such temperature shifts or to other environmental stresses, bacteria (Yamamori *et al.* 1978), yeast (McAlister *et al.* 1979), mammalian cells (Corces *et al.* 1981; Mirault *et al.* 1982; Pelham and Bienz 1982), and other cell types (Ashburner and Bonner 1979; Craig *et al.* 1982; Kelley and Schlesinger 1978; Pelham and Bienz 1982; Voellmy and Rungger 1982) produce a set of proteins

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which seem to be highly conserved through evolution (Craig *et al.* 1982) and which function to protect the cells from the toxic effects of such stresses (Ashburner 1982). The onset of the synthesis of these heat shock proteins is rapid and, in some cases, accompanied by a concomitant decrease in the synthesis of proteins normally produced (Ashburner 1982; Ashburner and Bonner 1979; Craig *et al.* 1982; Duncan and Hershey 1984; McKenzie *et al.* 1975).

Differences in protein composition, especially in protein surface components, may be expected in forms of an organism found in very different environments in nature. This is true for the promastigote and amastigote forms of leishmania. Immunochemical analyses of promastigotes and amastigotes of several *Leishmania* species have resulted in the identification of stage-specific and common protein antigens (Handman and Curtis 1982; Handman *et al.* 1984; Smejkal *et al.* 1984; Sadick and Raff 1985; Pan 1986). In general, a comparison by these investigators of the total or surface complement of protein antigens has revealed mostly shared, and only a few (3–7) stage-specific, antigens; some antigens, although shared, were found to be predominantly expressed in either promastigotes or amastigotes. During the intracellular transformation of *L. mexicana* from promastigotes to amastigotes in macrophages, changes have been observed mostly in protein bands ranging from M_r 24,000 to 68,000 (Chang and Fong 1982). Several proteins specific to the amastigote have been postulated to provide protection against the hostile environment of the phagolysosome (Handman and Greenblatt 1977; Gottlieb and Dwyer 1981) and to play a role in the increased survival rate of amastigotes in macrophages (Mauel 1984).

Several groups (Hunter *et al.* 1982, 1984; Hansen *et al.* 1984; Smejkal *et al.* 1984; Van der Ploeg *et al.* 1985) have described an *in vitro* temperature-induced conversion of the promastigote of various *Leishmania* spp. to a round, aflagellated, extracellular form resembling the amastigote. Known variously as an axenic amastigote (Hansen *et al.* 1984; Smejkal *et al.* 1984), a culture-derived amastigote (Hunter *et al.* 1982), a differentiating form (Hunter *et al.* 1984), and a high-temperature promastigote (Van der Ploeg *et al.* 1985), this form is obtained from promastigotes in culture by raising the temperature from 26 to 34 C. During temperature-induced differentiation, seven actively synthesized proteins (underexpressed or absent from the promastigote) have been identified with M_r's 83,000, 70,000, 68,000, 27,000, 23,000, and 22,000 (Hunter *et al.* 1984). These proteins correspond in molecular weight to a small set of proteins, termed Hsps; the synthesis of these Hsps has been found to be induced as a response to a heat shock or stress situation in every eukaryotic and prokaryotic organism thus far examined (Bienz 1985). Indeed, sequences related to cloned heat shock genes, Hsp70 and Hsp83, from *Drosophila* have been detected in the nuclear DNA of *L. major* with hybridization analyses producing similar patterns for the lesion amastigotes and the differentiating high-temperature promastigote (Van der Ploeg *et al.* 1985).

However, to our knowledge, there has been no evidence that these responses and changes reflect development of promastigotes into an infective stage. We now report that, concomitant with changes in morphology and protein composition, the infectivity of heat-shocked leishmania promastigotes greatly exceeds that of promastigotes.

Materials and Methods

Leishmania braziliensis panamensis, stock WR470, was originally obtained from a patient presenting at the Walter Reed Army Medical Center with cutaneous leishmaniasis. Promastigotes were grown at 26 C in Schneider's Drosophila Medium (Biofluids) containing 25% fetal bovine serum (GIBCO) and 50 mg gentamycin/liter of medium unless otherwise stated. Fresh stabilates from the same freeze-down were defrosted for each experiment.

To convert promastigotes to heat-induced differentiating forms, log-phase promastigote cultures were washed extensively in PBS, counted, and aliquotted into separate flasks in fresh Schneider's medium at $5-10\times10^7$ cells/ml. Those cultures to be converted were then transferred to 34 C, while control cultures were maintained at 26 C. In our hands, the parasites remained just as viable and converted just as well in Schneider's medium as in Medium 199 as described by Hansen *et al.* (1984). Carrying out the conversion in Schneider's medium allowed for a more direct comparison between cells grown at 26 and 34 C, controlling for any possible artifacts generated by growing the cells in two different media.

At time 0 and at each time point thereafter, an aliquot of each culture was counted and checked for viability and morphological type by the method of Jackson *et al.* (1985) using a Zeiss microscope equipped with uv optics. This test for viability is a double dye technique, using ethidium bromide and fluorescein diacetate. The diacetate is metabolized in viable cells to yield the green fluorescing dye, while dead cells emit the red color characteristic of ethidium bromide. Samples of culture were harvested by centrifugation, washed, and then suspended in PBS to approximately the same cell density ($5-10\times10^7$/ml). An aliquot for animal infections was removed from each sample and the remainder of the cell suspension was centrifuged and stored as a dry pellet at -70 C until all samples were ready for analysis by PAGE.

The infectivity of each sample was tested in adult (age 5 months) male Syrian hamsters (Charles River). Between 24 and 48 hr prior to infection, the hamsters were shaved at the base of the tail. Parasites from the aliquots reserved for infecting were centrifuged and suspended to approximately 5×10^8 cells/ml in Medium 199. One animal was mock infected by the injection of Medium 199 alone. Three animals per experimental condition were injected intradermally at the base of the tail with 30 μl Medium 199 containing $2-5\times10^7$ parasites. The 2.5-fold difference in parasite number is within acceptable limits to ensure reproducibility of results (Pan and Honigberg 1985, unpublished observations). The animals were checked periodically for appearance of a papule or lesion, and the affected area was measured.

The protein composition of cells at each time point was analyzed by SDS-PAGE according to the method of Laemmli (1970). Pellets, each containing 10^9 organisms, were solubilized in SDS sample buffer (2% SDS, 5% mercaptoethanol, 2% Nonidet-P40, 10% glycerol, and 0.001% bromphenol blue). Extracts were heated at 95 C for 3 min, loaded onto a 0.75-mm-thick slab gel containing a 6% stacking gel and a 12.5% separating gel, and then electrophoresed at constant voltage. Gels were silver stained using the method of Morrissey (1981).

RESULTS

Promastigotes of *Leishmania braziliensis panamensis*, strain WR470, were converted to heat-induced differentiating forms by transferring culture to 34 C. The cultures were monitored at various time intervals by sampling an aliquot immediately before harvesting. A cell count was obtained, and the cells were observed for viability, morphology, and motility. As shown in Fig. 1 and Table I, promastigotes cultured at 26 C remained elongated and motile throughout the course of the experiment. The number of viable cells dropped slightly by the end of 120 hr, probably due to a depletion of nutrients, since cultures were not supplemented during the experiment. The cells shifted to 34 C began to change their morphology between 2 and 5 hr, and by 9 hr, most had become round and amotile, resembling amastigotes. Although the percentage of viable cells at 48 hr was slightly less in the culture grown at 34 C than in the one grown at 26 C, the viability of both cultures was essentially the same by 120 hr.

The infectivity of the parasites at various stages in the conversion of promastigote to heat-induced differentiating form was determined by injecting a suspension of parasites into a susceptible animal at various time points and observing the appearance and size of resulting papules or lesions. The results are shown in Table II. The inoculum obtained at time 0 produced a papule or lesion by 40 days after infection. The size of the affected area grew quickly to 9.5–11 mm and was evident for 60 days. Essentially, the same infectivity was manifested by cells grown for 2 hr at 26 C. However, after 2 hr growth at 34 C, the inoculum caused a papule or lesion which appeared 4 days earlier, grew to almost twice the size of that produced by the cells from time 0 or 2 hr at 26 C, and lasted 84 days. The size of the affected area and the length of time it was observed continued to in-

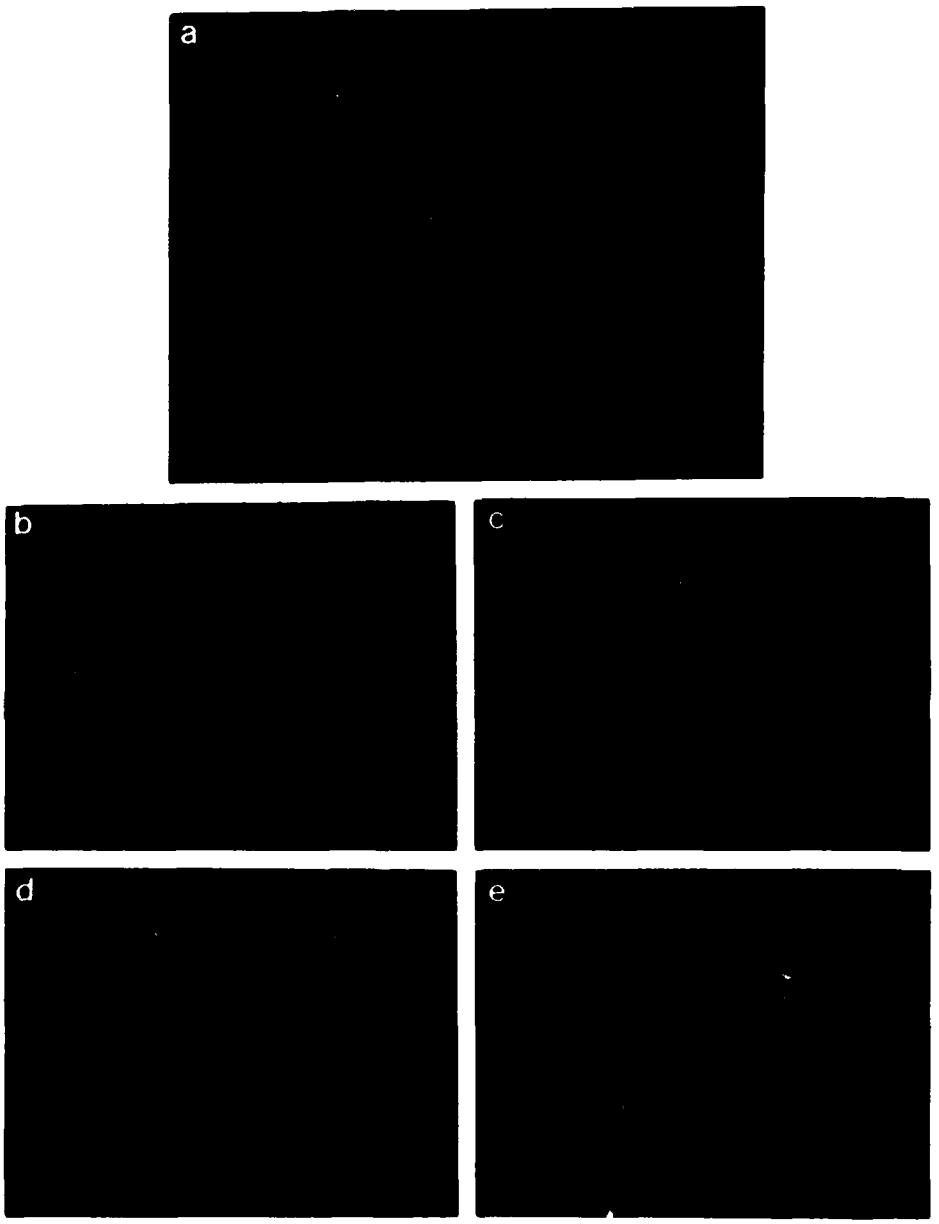

FIG. 1. Fluorescence micrographs of *Leishmania braziliensis panamensis* promastigotes and organisms undergoing temperature-induced conversion. (a) Promastigotes after 48 hr at 26 C. The cells retain the morphology they had at time 0; (b) 2 hr at 34 C; (c) 4 hr at 34 C; (d) 24 hr at 34 C; (e) 48 hr at 34 C.

TABLE I
Conversion of Promastigotes to Heat-Induced Differentiating Forms

Time (hr)	Temperature (C)	% Viable cells	Morphology
0	26	>99	Slender, motile
2	26	>99	Slender, motile
2	34	>99	Slender, motile
5	34	94.1	50% Round and amotile
9	26	>99	Slender, motile, and clumped
9	34	96.7	>75% Round and amotile
24	26	>99	Slender, motile, and clumped
24	34	95.7	Round, amotile
48	26	93.5	Slender, motile, and clumped
48	34	87.2	Round, amotile, and clumped
120	26	82.2	Slender, motile, and clumped
120	34	81.3	Round, amotile, and clumped

Note. Promastigotes in log phase culture were aliquotted into flasks, which were maintained at 26 C or transferred to 34 C. At the times indicated, a flask from each temperature was sampled and observed for viability and morphology.

crease throughout the experiment, and after 120 hr, the infectivity of both cultures was about equal.

The protein composition of the cells at each sampling time was analyzed by PAGE (Fig. 2). The protein composition of the cells in culture at 26 C showed few qualitative and only some quantitative changes until 120 hr, at which point some notable changes were observed. In contrast, the cultures transferred to 34 C showed a dramatic decrease in the number of proteins present as the time at the elevated temperature increased, a phenomenon observed in other systems, as well (Craig *et al.* 1982; Ashburner and Bonner 1979; Ashburner 1982; McKenzie *et al.* 1975; Duncan and Hershey 1984). Bands at M_r 84,000 and M_r 81,000 (c, d) had decreased by 48 hr and were gone by 120 hr at 34 C. Also notable was the disappearance of a number of bands below M_r 40,000. For example, after 9 hr at 34 C, bands at M_r 38,000 and M_r 36,000 (o, p) began disappearing. Another band at M_r 29,000 (r) decreased in intensity after 24 hr at 34 C. A band at M_r 25,000 (t)

TABLE II
Infectivity of Promastigotes and Heat-Induced Differentiating Forms

Sample (hr/°C)	Time of appearance (days after infection)	Time of maximum size (days after infection)	Maximum size (mm)	Duration (days)
0	40	48–70	9.5–11	60
2/26	40	48–66	10–12	50
2/34	36	50–73	17–18.5	84
9/26	36	48–70	12–15	64
9/34	34	51–71	20–21	86
48/26	35	47–65	17.5–20	90
48/34	33	48–70	25–32	92
120/26	32	41–67	22–28	93
120/34	32	42–61	22–28	93

Note. Cells at each timepoint were injected intradermally into Syrian hamsters at the base of the tail. The animals were checked periodically for appearance of a papule or lesion, and the affected area was measured.

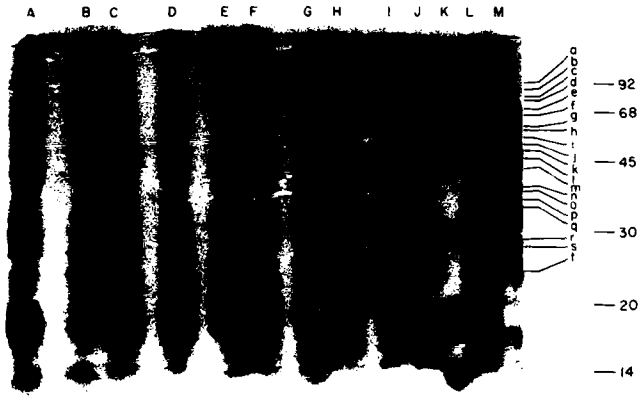

FIG. 2. Electrophoretic patterns of samples taken from cultures grown at 26 or 34 C at various time points. (A) Time 0; (B) 2 hr at 26 C; (C) 2 hr at 34 C; (D) 5 hr at 34 C; (E) 9 hr at 26 C; (F) 9 hr at 34 C; (G) 24 hr at 26 C; (H) 24 hr at 34 C; (I) 48 hr at 26 C; (J) 48 hr at 34 C; (K) Molecular weight standards; (L) 120 hr at 26 C; (M) 120 hr at 34 C.

was one of the few prominent bands in this region after 120 hr at 34 C. This band, however, was present in both culture systems after 9 hr at their respective temperatures. The band was more prominent in the 26 C samples and persisted with equal intensity throughout the time frame of incubation. A weaker, but similarly persistent, band was noted for the 34 C samples.

Protein bands at M_r's 61,000, 59,000, 55,000, 52,000, 49,000, 46,000, 43,000, and 39,000 (g–n, respectively) were present in all samples, but some were more abundant in the later 120-hr, 34 C samples. The M_r 55,000 and M_r 52,000 bands most likely represent the α and β subunits of tubulin. These bands were more prominent in the 26 C samples but markedly decreased in intensity at 120 hr; 34 C samples began to show a progressive decrease beginning at 9 hr. During the course of the experiment, a number of bands at M_r's 93,000, 87,000, 72,000, 68,000, and 28,000 (a, b, e, f, s) were observed earlier or were more prevalent in the 34 C samples. A doublet band at M_r 36,000 and M_r 35,000 (p, q), appearing in early 26 and 34 C samples, maintained its M_r 35,000 intensity but gradually lost the discrete M_r 36,000 component at 26 C; at 34 C both bands weakened considerably with successive sampling, disappearing at 48 hr with only the M_r 35,000 component strongly reappearing at 120 hr.

DISCUSSION

Leishmania spp. promastigotes transform into intracellular amastigotes after being transmitted by the sandfly to the warmer environment of the mammalian host. Amastigotes have a greater infectivity for experimental animals than do promastigotes, possibly due to their increased ability to survive the defense mechanisms of the host (Mauel 1984). Like true amastigotes, the infectivity of *L. braziliensis panamensis* heat-induced differentiating forms also appeared to be greater than that of promastigotes. Upon culture at 34 C, promastigotes of *L. b. panamensis* changed in morphology to that resembling intracellular amastigotes. However, by 2 hr after transfer to 34 C and even before the change in morphology began, the infectivity of the cells had increased (Tables I and II).

The heat shock phenomenon has been implicated in the transformation of promastigotes to heat-induced differentiating forms by Hunter *et al.* (1982, 1984) and confirmed by Van der Ploeg *et al.* (1985), who demonstrated sequence homology be-

tween the nuclear DNA of *L. major* and the *Drosophila* heat shock genes. These genes were found to be similar in the mammalian and high-temperature culture forms of the parasites. In many organisms, the transcriptional activation of heat shock genes in response to an increase in temperature causes the rapid appearance of heat shock proteins. In some case, as these genes are activated, others coding for proteins normally produced are turned off, causing a decrease in synthesis of the latter proteins (McKenzie *et al.* 1975; Kelley and Schlesinger 1978; Ashburner and Bonner 1979; Ashburner 1982; Craig *et al.* 1982; Voellmy and Rungger 1982; Duncan and Hershey 1984). The decrease in intensity of protein bands progressing to eventual disappearance and the predominance of a small set of proteins (M_r 28,000 to 87,000), coupled with the rapid change in infectivity when promastigotes in culture were transferred to 34 C, supports the involvement of a heat shock response that may promote differentiation into a more infective form or stage similar to the amastigote. It is reasonable to assume that the shift in temperature not only activates heat shock genes to produce traditional Hsps but may also trigger the production of stage-specific proteins, such as those described by us (Smejkal *et al.* 1984) and by Sadick and Raff (1985). Certain of these proteins, particularly if surface membrane-associated, may play an important role in parasite infectivity or virulence. In this regard, the increased infectivity and morphologic change likely reflect the alterations in the total protein profiles with one or more newly or predominantly synthesized proteins becoming responsible for or involved in the developmental differentiation leading to an increase in virulence. The identity of such proteins and their presumed role in developmental changes have yet to be demonstrated. It is likely that the stimulus resulting in increased infectivity is concurrently the trigger for synthesis of those proteins which appear as changes in the electrophoretic pattern (Fig. 2) beginning at 9 hr after transfer and which may be responsible for the change in morphology coinciding with their appearance. One or more of these proteins may also be responsible for the increase in infectivity. The differences in the electrophoretic patterns produced by cells cultured at 34 C and those at 26 C are particularly significant in view of the observed changes in infectivity and the evidence that infectivity may be dependent on the ability of surface components to protect the parasite from host defense mechanisms (Mauel 1984).

Recently, heat shock has been shown to trigger the induction of sexual reproduction in *Volvox*, the females of which otherwise reproduce asexually (Kirk and Kirk 1986). The rapid conversion by heat shock of one cell type to another in other organisms is, thus, probable. Developmental processes occurring during oogenesis may also activate heat shock genes (Bienz 1985). In contrast to the rapid response of heat shock genes to environmental stress, the developmental activation process appears to be slow. Increased attention is currently being given to both types of heat shock responses in organisms whose life cycles take them from an environment that is temperature regulated to one that is not. Both mechanisms of activation may occur during the life cycle of leishmania.

Developmental activation of some heat shock genes may occur during the "maturation" process of promastigotes in the insect vector. Promastigotes of *Leishmania tropica* were found to be more infective when taken from stationary phase cultures or from sandflies 7 to 10 days after a blood meal than from cultures growing logarithmically or from sandflies 3 days after a blood meal (Sacks and Perkins 1984). We have shown that *L. b. panamensis* promastigotes, while maintaining a similar morphology over the course of 120 hr, increased in infectivity with time in culture at 26 C (Tables I and II). Injection of promastigotes into a warm-blooded host by the

sandfly may trigger a heat-induced conversion similar to that produced in culture, where, in response to an increase in temperature, leishmania promastigotes undergo a conversion to heat-induced differentiating forms. Macrophages may then engulf the parasites already in the process of converting. Whether the changes that occur in the promastigote in culture as it transforms into the high-temperature form indeed mimic those that occur in a natural infection is unknown since an *in vivo* stage intermediate to the promastigote and amastigote has not yet been identified. However, the high-temperature differentiating forms will be of use in studying the promastigote to amastigote transformation and concomitant changes in infectivity and protein composition.

ACKNOWLEDGMENTS

We thank William M. Schweitzer for excellent technical assistance. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23.

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